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CRISPR interference (CRISPRi) as transcriptional repression tool for *Hungateiclostridium thermocellum* DSM 1313

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Summary

***Hungateiclostridium thermocellum* DSM 1313 has biotechnological potential as a whole-cell biocatalyst for ethanol production using lignocellulosic renewable sources. The full exploitation of *H. thermocellum* has been hampered due to the lack of simple and high-throughput genome engineering tools. Recently in our research group, a thermophilic bacterial CRISPR–Cas9-based system has been developed as a transcriptional suppression tool for regulation of gene expression. We applied ThermoCas9-based CRISPR interference (CRISPRi) to repress the *H. thermocellum* central metabolic lactate dehydrogenase (*ldh*) and phosphotransacetylase (*pta*) genes. The effects of repression on target genes were studied based on transcriptional expression and product formation. Single-guide RNA (sgRNA) under the control of native intergenic 16S/23S rRNA promoter from *H. thermocellum* directing the ThermodCas9 to the promoter region of both *pta* and *ldh* silencing transformants reduced expression up to 67% and 62% respectively. This resulted in 24% and 17% decrease in lactate and acetate production, correspondingly. Hence, the CRISPRi approach for *H. thermocellum* to downregulate metabolic genes can be used for remodelling of metabolic pathways without the requisite for genome engineering. These data established for the first time the feasibility of employing CRISPRi-mediated gene repression of metabolic genes in *H. thermocellum* DSM 1313.**

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Introduction

Microbial conversion of lignocellulosic renewable feedstock to fuels, chemicals and other bio-based products significantly reduces the dependence on non-sustainable fossil energy-based processes. This contributes to the transition towards a bio-based economy. Non-model production microbes with native ability of solubilizing lignocellulose have been improved for robust industrial production processes (Akinosho *et al.*, 2014; Lynd *et al.*, 2016; Kumar and Sharma, 2017). The anaerobic thermophilic bacterium *Hungateiclostridium thermocellum* is proficient at solubilizing cellulose as it possesses a hydrolytic multienzyme complex called the cellulosome. This enzyme complex solubilizes cellulose to cellodextrins that are further metabolized to produce ethanol, acetate, L-lactate, formate, hydrogen gas and carbon dioxide (McBee, 1954). Cellulose solubilization has the potential to improve product formation but not cost-effective for the conversion of lignocellulose to ethanol. Consolidated bioprocessing (CBP) combines enzyme production, cellulose hydrolysis and fermentation into a single step, offering an economical advantage to the current multistep process (Jiang *et al.*, 2017). In CBP no exogenous hydrolytic enzymes are added, thereby reducing the costs. Moreover, pretreatment, hydrolysis and fermentation steps are combined in one vessel, reducing the number of unit operations (Bhalla *et al.*, 2013). Besides, fermentation efficiency is improved as it also lowers the cost of the production of biofuels and biochemicals. To this end, the efficient genetic engineering of *H. thermocellum* would render it a highly valuable 'microbial cell factory' for biotechnological exploitation.

Metabolic engineering of *H. thermocellum* is necessary to improve the yield of ethanol or alternative preferred products from mixed-acid fermentation. However, the current genetic toolbox for *H. thermocellum* is still very limited. A plasmid-based homologous recombination gene disruption method, including *hpt* and *pyrF*-based counter selection, has been developed (Tripathi *et al.*, 2010). Using *hpt* and *pyrF* as a counter-selection marker

few genes such as *pta, ldh*, *adhE*, *cipA*, *cel48S, spooA* and *recA* were deleted to interrogate the genetic base for specific phenotypes and to create modified strains for different functions (Olson *et al*., 2010; Argyros *et al*., 2011; Brown *et al*., 2011; Olson *et al*., 2013; van der Veen *et al*., 2013; Groom *et al*., 2018; Lo *et al*., 2019). This tool has not been used extensively, as it is a laborious and time-consuming process. Subsequently, a Targetron system derived from the thermophilic cyanobacterium *Synechococcus elongatus* has been developed into a genome engineering tool for gene disruption in thermophiles, specifically *H. thermocellum* (Mohr *et al*., 2013). Targetron is a genome editing technology derived from mobile group II introns, which efficiently creates site-specific integrations for gene knockout and knock-in clostridia. The main limitations for this system include the inability to generate clean knockouts, reduced target specificity due to lack of intron-encoded protein (IEP) recognition sites for small genes, the variation in the intron-integration efficiency and the presence of ectopic intron insertion events (Song *et al*., 2015; Bruder *et al*., 2016; Joseph *et al*., 2018).

The development of simple, and preferably high-throughput, genome engineering tools is crucial for efficient metabolic engineering, as well as for a full exploitation of this thermophile. The CRISPR–Cas9 technology has provided a precise technique for genome editing, paving the way for a new era in molecular biology. In addition, CRISPRi has commenced as an effective technique for gene downregulation and can be used to modulate gene expression. This approach uses a nuclease-deficient Cas9 (dCas9) in conjunction with spacer region of a single-guide RNA to repress specific genes targeting at the promoter and start of the gene. CRISPRi systems have been established in mesophilic clostridial species, including *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Li *et al*., 2016; Wang *et al*., 2016; Wen *et al*., 2017). Recently, a thermophilic Cas9, ThermoCas9, has been isolated from the thermophilic bacterium *Geobacillus thermodenitrificans* T12 (Daas *et al*., 2018). It is a type-IIC Cas9 that is active over a broad temperature range. A dCas9 variant has been generated and used for CRISPRi silencing of the lactate dehydrogenase activity in the thermophilic *Bacillus smithii* (Mougiakos *et al*., 2017).

High repression of gene expression has been reported when the dCas9-sgRNA ribonucleoprotein complex targets the non-coding DNA strand (5'–3') of a gene, specifically close to the ATG start codon, whereas the effect is less pronounced if the coding strand (3'–5') is targeted. Furthermore, effective gene silencing is observed when targeting the promoter region, with the strongest effect at the −35 sequence, being independent of the DNA strand (Qi *et al*., 2013). It has been hypothesized that the inhibition of the transcription is due to a physical collision between the RNA polymerase and the dCas9-sgRNA complex. During elongation, the RNA polymerase encounters the dCas9-sgRNA, which is bound to the non-template DNA, pausing the transcription elongation. Alternatively, if dCas9-sgRNA complex is bound to the template strand, slight repressive effect can be observed as the RNA polymerase could still read through the complex. The sgRNA faces the RNA polymerase, which could unzip the complex by helicase activity upon targeting the template strand (Qi *et al*., 2013). Moreover, gene knockdowns are less likely to be lethal and frequently permit analysing surviving mutants where drastic knockout techniques fail (Qi *et al*., 2013; Pyne *et al*., 2014; Peters *et al*., 2016).

In this study, ThermodCas9 was adapted for use in *H. thermocellum* as a tool to enable silencing of gene expression. The CRISPRi system was implemented to effectively knockdown metabolic genes such as lactate dehydrogenase (*ldh*) and phosphotransacetylase (*pta*) via appropriate sgRNA design. Selective repression of the *ldh* and *pta* genes decreased lactate and acetate production respectively. Hence, demonstrating the feasibility of employing CRISPRi for the metabolic engineering of *H. thermocellum* and production of bio-derived chemicals.

Results

Selection of promoters

To establish the ThermodCas9-based CRISPRi tool in *H. thermocellum*, functional promoters are required for efficient expression of the ThermodCas9 and the sgRNA. The pThermoCas9i plasmid (Mougiakos *et al*., 2017) encodes the catalytically inactive variant of ThermoCas9 (ThermodCas9) and the non-targeting sgRNA-expressing module under the control of the constitutive *xylL* promoter from *B. smithii* and *pta* promoter from *Bacillus coagulans* respectively. This plasmid was introduced in *H. thermocellum* DSM 1313 via electroporation as described by Olson *et al*. (Olson and Lynd, 2012). To validate expression of both genes, RNA was isolated from *H. thermocellum* transformants containing the non-targeting pThermoCas9i plasmid that were grown in CP medium. RT-PCR on cDNA showed that the ThermodCas9 gene was expressed, but the sgRNA was not expressed (Fig. 1). From this, we concluded that the *xyl*L promoter from *B. smithii* is functional in *H. thermocellum*, but the *pta* promoter from *B. coagulans* is not.

Promoter screening for the establishment of CRISPRi system in H. thermocellum *DSM 1313*

Based on the previous results, it was necessary to replace the *B. coagulans pta* promoter with an

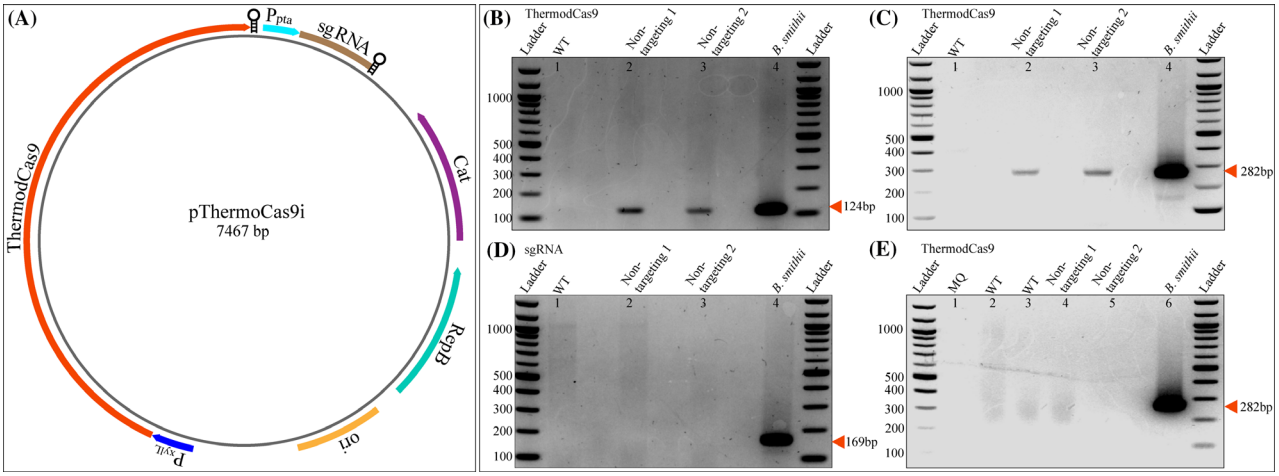

Fig. 1. RT-PCR of viable *H. thermocellum* transformants, containing the pThermoCas9i plasmid. A. Schematic illustration of plasmid pThermoCas9i_NT (Mougiakos *et al*., 2017). Thermodcas9 gene under control of the *B. smithii xylL* promoter; sgRNA-expressing module under control of the *B. coagulans pta* promoter; and pNW33n backbone. B, C and D. Non-targeting 1 and 2, independent pThermoCas9i_NT (Mougiakos *et al*., 2017) transformants of *H. thermocellum* (lanes 2 and 3) showing the RT-PCR products of 124 bp (B) and 282 bp (C) from thermodcas9 cDNA and the absence of the RT-PCR product of 169 bp (D) from the sgRNA; WT, RT-PCR with *H. thermocellum* DSM 1313 wild-type cDNA; *B. smithii*, RT-PCR with *B. smithii* harbouring pThermoCas9i_NT (Mougiakos *et al*., 2017) cDNA. E) MQ, RT-PCR with Milli-Q water (lane 1); WT, RT-PCR with *H. thermocellum* DSM 1313 wild-type DNA (lane 2); WT, RT-PCR with *H. thermocellum* DSM 1313 wild-type RNA (lane 3); Non-targeting 1 and 2, RT-PCR with *H. thermocellum* transformants 1 and 2 RNA, respectively, (lane 4 and 5); and *B. smithii*, RT-PCR with *B. smithii* harbouring pThermoCas9i_NT (Mougiakos *et al*., 2017) cDNA (lane 6).

alternative one to efficiently express the sgRNA. To develop an efficient system, we decided to assess the use of native promoter of small ribosomal intergenic 16S/23S rRNA from *H. thermocellum*. Due to the limited number of characterized promoters from this anaerobic thermophile, we selected approximately 250 bp long upstream sequence of the 16S and 23S rRNA genes anticipating the presence of promoters (Appendix S1). Ribosomal promoters are preferred for efficient sgRNA expression (Li *et al*., 2013; Xu *et al*., 2014). We replaced the *pta* promoter of the initial constructed plasmids with the predicted intergenic 16S/23S rRNA promoters and tested sgRNA expression by RT-PCR (Fig. 2). The intergenic 16S/23S rRNA promoter drove sgRNA gene expression. Therefore, we chose the *xylL* promoter to drive ThermodCas9 gene expression and the 16S/23S rRNA promoter to drive sgRNA expression.

*Transcriptional repression on lactate dehydrogenase (*ldh*) and phosphotransacetylase (*pta*) genes*

To investigate the utility of CRISPRi for gene repression in *H. thermocellum*, we aimed to target the non-template strand of both lactate dehydrogenase (*ldh*) and phosphotransacetylase (*pta*) genes. We constructed pThermoCas9i vectors with the *xylL* promoter expressing the ThermodCas9 and the intergenic 16S/23S rRNA promoter controlling the sgRNA together with a non-targeting spacer (pThermoCas9i_NT) or a spacer targeting the non-template DNA strand overlapping either the promoter region or the start of the gene for both *ldh* (*ldh*_P; *ldh*_S) and *pta* (*pta*_P; *pta*_S) genes respectively. Targeting and non-targeting plasmids were electroporated to *H. thermocellum* DSM 1313. A drop of two orders of magnitude in transformation efficiency was observed when using the targeting plasmid (10^2 CFU μg DNA^{-1}) in comparison with the controls (10^4 CFU μg DNA^{-1}). All the tested colonies for the targeting plasmid showed a positive band for the expression of the ThermodCas9 and sgRNA. These clones were grown in 50 ml CP medium containing 6 μg ml^{-1} thiamphenicol (Tm^R) and cultured to OD_{600} ~ 0.6 and > 1.0. Five millilitres of cells were sampled for RNA extraction and qRT-PCR analysis to determine the downregulation of the silencing transformants. qRT-PCR was performed using *gyrA* and *recA* as house-keeping genes. Eventually, *recA* was used as a reference gene as it appeared more consistent in expression than *gyrA* (data not shown).

The qRT-PCR analysis (Fig. 3) on RNA isolated from exponentially growing cells harvested at an OD_{600} ~ 0.6 showed 62 % and 58% reduction in *ldh* gene expression, using the expression in control transformant cells with the pThermoCas9i_NT (NT control) as the reference, when targeting the promoter (*ldh*_P) and start of the gene region (*ldh*_S) respectively. When harvested at the early stationary phase at an OD_{600} > 1.0, the repression effect was still retained 43% for the *ldh*_P, but the *ldh*_S transformant no longer showed repression and had the same expression level as the NT control. Similarly, at an OD_{600} ~ 0.6 for the *pta*-targeting

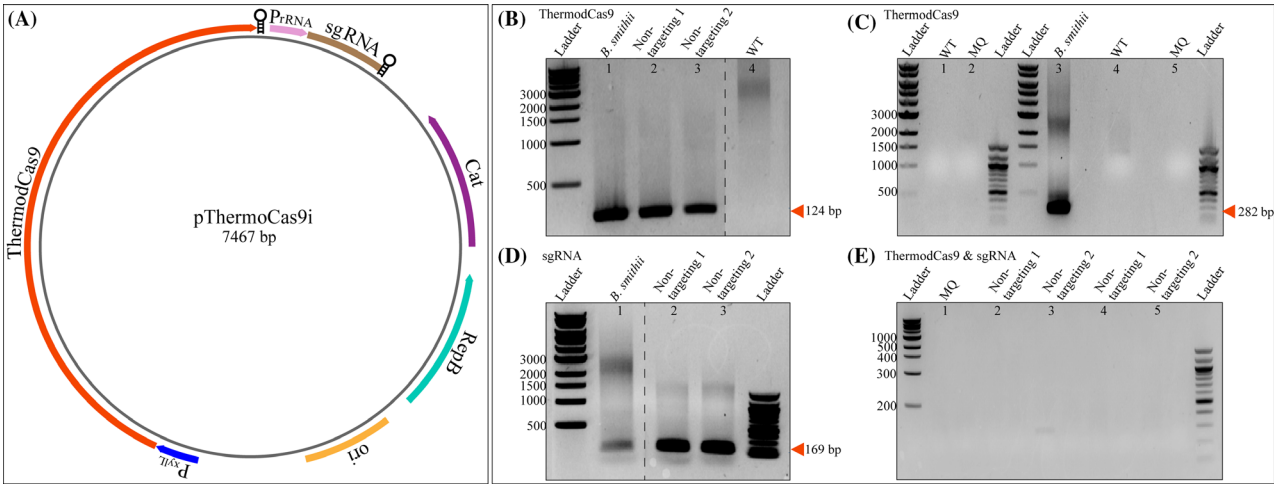

Fig. 2. RT-PCR of viable *H. thermocellum* transformants, containing the pThermoCas9i plasmid with rRNA promoters. A. Schematic illustration of plasmid pThermoCas9i_NT (Mougiakos *et al.*, 2017). The *thermodcas9* gene under control of the *B. smithii xylL* promoter; sgRNA-expressing module under control of the native rRNA promoter; and pNW33n backbone. B. Non-targeting 1 and 2, independent pThermoCas9i_NT (Mougiakos *et al.*, 2017) transformants of *H. thermocellum* (lanes 2 and 3) showing the RT-PCR product of 124 bp from *thermodcas9* cDNA; WT, RT-PCR with *H. thermocellum* DSM 1313 wild-type cDNA; *B. smithii*, RT-PCR with *B. smithii* harbouring pThermoCas9i_NT (Mougiakos *et al.*, 2017) cDNA. C. WT, RT-PCR with *H. thermocellum* DSM 1313 wild-type DNA (lane 1 and 4); MQ, RT-PCR with Milli-Q water (lane 2 and 5); and *B. smithii*, RT-PCR with *B. smithii* harbouring pThermoCas9i_NT cDNA (lane 3). D. Non-targeting 1 and 2, independent pThermoCas9i_NT (Mougiakos *et al.*, 2017) transformants of *H. thermocellum* (lanes 2 and 3) showing the RT-PCR product of 169 bp from the sgRNA. E) RT-PCR with the RNA from non-targeting 1 and 2, independent pThermoCas9i_NT (Mougiakos *et al.*, 2017) transformants of *H. thermocellum.*

transformants the silencing effect was 67% and 62% when targeting the promoter (*pta*_P) and start of the gene (*pta*_S) respectively. When the $OD_{600} > 1.0$, the expression was 45% and 39% reduced for the *pta*_P and *pta*_S transformants respectively. These data indicate that CRISPRi suppressed the gene expression, and the silencing efficacy was high in comparison to the control.

In addition, the impact of the silencing on the other respective genes involved in product formation was evaluated. At $OD_{600} \sim 0.6$, the *ldh* gene for *pta* silencing transformants targeting the promoter (*ldh*_E1) and start of the gene (*ldh*_E2) showed enhanced expression respectively compared with the NT control. For the early stationary phase cultures, the *ldh*_E1 expression was same as NT control but increased for the *ldh*_E2. Contrarily, the *pta* gene had reduced expression compared with the NT control at early exponential phase, for both the *ldh* silencing transformants (*pta*_E1, targeting the promoter and *pta*_E2, targeting the start of the gene). Hence, the non-targeted metabolic *pta* and *ldh* genes responded differently to the silencing of *ldh* and *pta* transformants respectively (data not shown).

Impact of gene silencing on product formation using HPLC

To evaluate the effect of repression of *ldh* and *pta* gene on organic acids production, silencing transformants were cultured to an $OD_{600} \sim 0.5–1.5$, in anaerobic bottles containing 50 ml CP medium (1 g l^{-1} yeast extract) for 2 days. Samples were taken at two time points for HPLC analysis for the two transformants in comparison with the NT control. Moreover, the fermentation profile was observed to see the effect of knockdown of *ldh* and *pta* genes on other organic acids and ethanol production.

It was clearly noted that repression of *ldh* and *pta* genes at the promoter region led to a 24% and 17% reduction on lactate and acetate production in comparison with NT cells respectively (Fig. 4). At time point T1 ($OD_{600} \sim 0.5–0.7$), the acetate production in the *pta* silencing transformants (*pta*_P and *pta*_S) was suppressed. In contrast, the lactate production for these transformants was increased around 25% to the NT control. For the *ldh* silencing transformants (*ldh*_P and *ldh*_S), lactate was reduced drastically with slender decrease in acetate as well. Other products for both *ldh* and *pta* silencing transformants such as formate had slightly better yield in all silencing transformants whereas ethanol did not show any significant differences from the control. When the $OD_{600} > 1.0$ (T2), the yield (mol of lactate/mol of cellobiose consumed) for *ldh*_P was 0.36 and *ldh*_S was 0.55 whereas that of NT was 0.48. Thus, lactate production was still low for the *ldh*_P transformant. Contrarily, both the *pta* silencing transformants had similar levels of acetate, formate and ethanol production compared with the NT control (Appendix S2, Fig. S1).

In summary, HPLC showed decrease in acetate and lactate production at an exponential phase but in the late

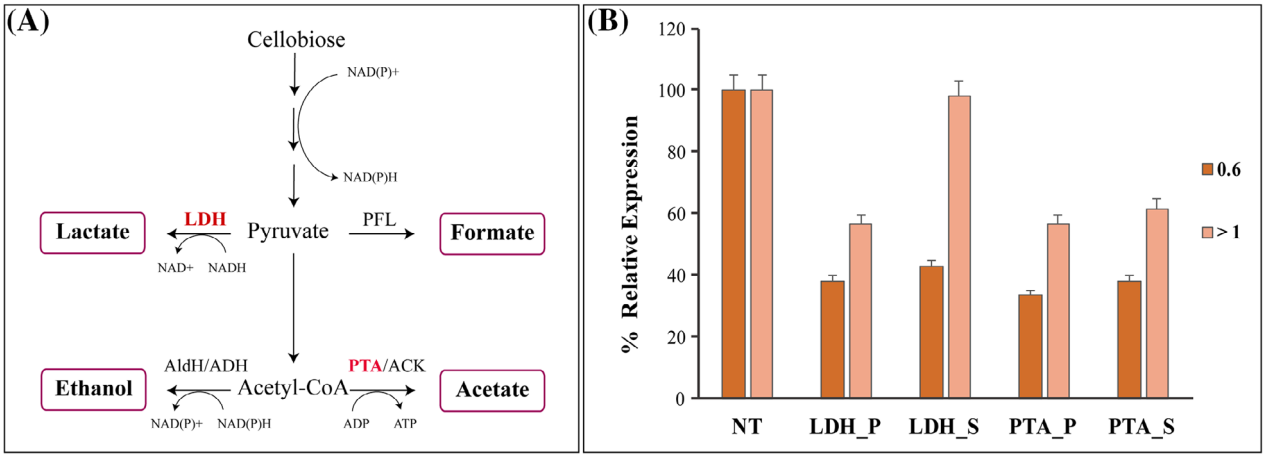

Fig. 3. Effective suppression of *ldh* and *pta* genes by CRISPRi. A. Metabolic pathway representation of *H. thermocellum* DSM1313 for conversion of cellobiose to ethanol and major organic acids production. (ACK, acetate kinase; ADH, alcohol dehydrogenase; AldH, aldehyde dehydrogenase; LDH, lactate dehydrogenase; PFL, pyruvate-formate lyase; and PTA, phosphotransacetylase). B. Relative *ldh* and *pta* gene expression was evaluated using qRT-PCR in comparison with control non-targeting (NT) at an OD_{600} – 0.6 and 1.0 respectively; positive silencing transformants for *ldh* gene referred as *ldh*_P – targeting the promoter, *ldh* _S – targeting the start of the gene; positive silencing transformants for *pta* gene referred as *pta*_P – targeting the promoter, *pta*_S – targeting the start of the gene. *H. thermocellum* DSM 1313 cells were transformed with both non-targeting pThermoCas9i_NT and targeting pThermoCas9i_*ldh*/*pta* plasmids. The Tm^R colonies of both the non-targeting and targeting plasmids were transferred to 50 ml CP (5 g l^{-1} yeast extract) medium containing 6 μg ml^{-1} Tm^R and cultured to OD_{600} ~ 0.6 and > 1.0, and 5 ml cells were sampled for qRT-PCR analysis with technical triplicates. The expression levels were normalized to those in the NT control. Data represent the mean values of three biological replicates and the standard deviation (SD). The level of significance of the differences when results were compared was estimated by means of analysis of variance (ANOVA), with $\alpha = 0.05$.

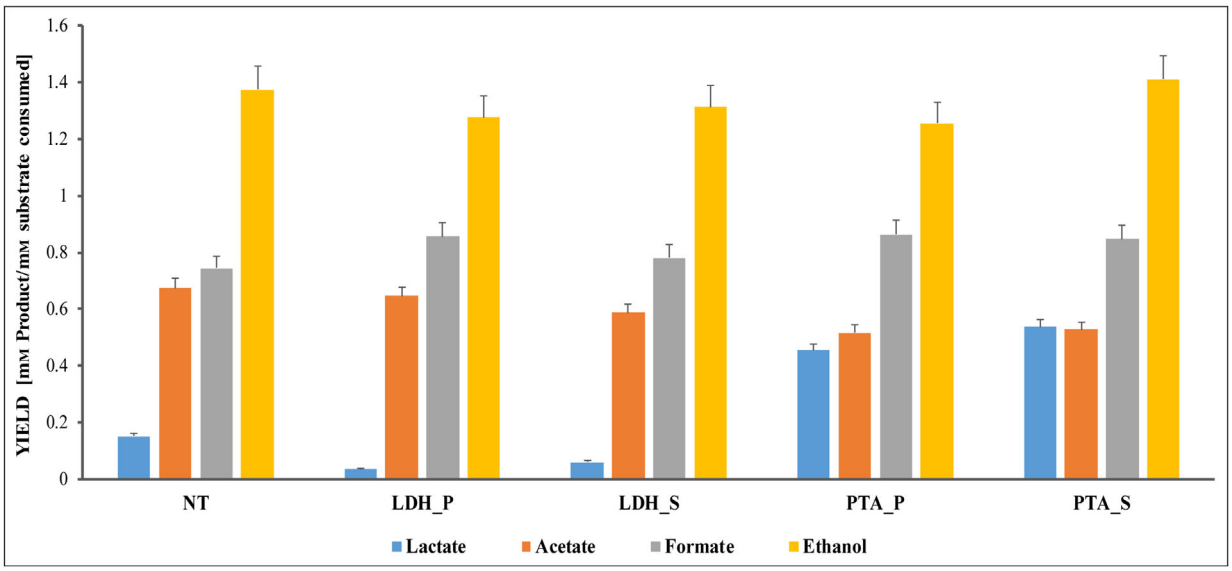

Fig. 4. Effects of CRISPRi-mediated suppression on lactate and acetate production and impact on the fermentation profile. For lactate and acetate production, positive transformants referred as *ldh*_P, *ldh* _S, *pta*_P, *pta*_S and control *ldh*_non-targeting (NT) were analysed in HPLC at OD_{600} – 0.5–0.7 (T1). The silencing transformants for *ldh* gene referred as *ldh*_P – targeting the promoter, *ldh* _S – targeting the start of the gene; positive silencing transformants for *pta* gene referred as *pta*_P – targeting the promoter, *pta*_S – targeting the start of the gene. *H. thermocellum* DSM 1313 cells were transformed with both non-targeting pThermoCas9i_NT and targeting pThermoCas9i_*ldh* and *pta* plasmids. The Tm^R colonies of both the non-targeting and targeting plasmids were transferred to 50 ml CP medium (1 g l^{-1} yeast extract) containing 6 μg ml^{-1} Tm^R and cultured to OD_{600} ~ 0.5–0.7 (T1) and > 1.0 (T2), and 1 ml cells were sampled for 2 days for HPLC analysis. Data represent the mean values of three biological replicates and the standard deviation (SD). The level of significance of the differences when results were compared was estimated by means of analysis of variance (ANOVA), with $\alpha = 0.05$.

stationary phase the fermentation products come to same yields as the NT cells. This shows that despite that ThermodCas9 can silence specific metabolic genes, *H. thermocellum* can escape the silencing upon prolonged incubation.

Discussion

CRISPRi offers reduction in gene expression and can be used to interrogate gene function and modulate cellular activities. Recent studies have explored CRISPRi to control the metabolic pathways in *E. coli* and other non-model organisms for improved production of numerous biotechnological products (Mougiakos *et al*., 2018). In this study, we applied the CRISPR-ThermoCas9-based transcriptional regulation tool for the first time an effective system in *H. thermocellum* DSM 1313. The existing methods for genome editing and transcriptional regulation purposes are laborious, regularly inefficient and technically complex in the anaerobic bacteria, which are known to be challenging for genetic manipulation. The recent discovery of a thermostable Cas9 coupled to its transformation into a powerful genetic tool has opened new possibilities for genome manipulation of the thermophilic bacteria (Mougiakos *et al*., 2017). The dCas9-based CRISPRi allows the transcriptional regulation of the gene of interest without completely disrupting its function, resulting in less pleiotropic effects than gene knockouts (Dominguez *et al*., 2016; Peters *et al*., 2016).

To evaluate the functionality of the CRISPRi tool in thermophilic clostridia, we targeted two genes of the central metabolism; the lactate dehydrogenase (*ldh*) and phosphotransacetylase (*pta*) in *H. thermocellum* DSM 1313. It has been proven that the deletions of these genes are not detrimental for its survival and allows the increase in ethanol production (Argyros *et al*., 2011; Biswas *et al*., 2014; Papanek *et al*., 2015). We constructed two ThermodCas9 expressing vectors, with sgRNA genes targeting the promoter and start of the gene plus a control plasmid with a non-targeting sgRNA. Based on previous studies, the target sites were located in the non-template strand, near the promoter region and start of both *ldh* and *pta* genes. According to literature, the highest transcriptional downregulation effect was expected to occur where ThermodCas9 targets the promoter or beginning of the coding sequence, the lowest repression in the transformants where the middle of the gene was targeted (Larson *et al*., 2013; Qi *et al*., 2013; Yao *et al*., 2016).

The first steps towards the adaptation of ThermodCas9-based CRISPRi tool showed the *xylL* promoter from *Bacillus smithii* allows the expression of ThermodCas9 protein in *H. thermocellum*. However, the *pta* promoter from *Bacillus coagulans* failed to transcribe the gene encoding the sgRNA. Bacilli and clostridia are closely related bacteria that belong to the phylum Firmicutes (Talukdar *et al*., 2015). The close-relatedness among these two classes might explain the functionality of the *xyl*L promoter from *Bacillus smithii* in *H. thermocellum*, specifically because both are thermophilic strains. *Bacillus coagulans* is also a facultative anaerobic thermophilic bacterium, but the reason for the non-functioning of the *pta* promoter in *H. thermocellum* remains unclear.

To develop an efficient CRISPRi tool, we successfully managed to get the activity of putative native intergenic 16S/23S rRNA promoter for the expression of the chimeric sgRNA. The promoter region within 16S/23S intergenic spacer sequence has homology to the prokaryotic consensus promoter structure. In *E. coli*, it has been shown that this promoter has a role in the transcription of ribosomal RNAs (Mankin *et al*., 1987; Zacharias and Wagner, 1989). Now, we show that this is also likely to be the case for *H. thermocellum*.

This study shows the first attempt to exploit the CRISPRi tool in thermophilic clostridia to modulate gene expression using the central metabolism phosphotransacetylase (*pta*) and lactate dehydrogenase (*ldh*) genes as examples. The results showed downregulation levels of 67% and 62%, respectively, when the promoter and the start of *pta* gene were targeted. Consequently, the organic acids production changed in comparison with NT control: acetate production decreased between 17% and 27%; formate production increased between 5% and 12%; and ethanol had no significant change. As studied before, the *pta* knockout mutant generated using the Targetron system had 13.5% and 81.5% decreased acetate and lactate production, respectively, and 42% improvement in ethanol production (Mohr *et al*., 2013). Contrarily, we observed 30% of increased lactate production and this correlated well with the upsurge in *ldh* gene expression for *pta* silencing transformants. Argyros *et al.* (2011) have shown similar impact of improved lactate titre with *pta* knockout. In addition, no significant increase in ethanol production was noted in comparison with the NT control. The plausible explanation for this could be despite of the silencing, the residual expression still resulted in sufficient enzyme activity for the cell to still produce acetate and ATP instead of ethanol and NAD(P). Besides, further genetic engineering of pyruvate metabolism is required to increase ethanol production as discussed in previous studies (Dash *et al*., 2017; Tian *et al*., 2017).

The *ldh* silencing transformants *ldh*_P and *ldh*_S similarly had 62% and 58% downregulation levels with decrease in lactate production in comparison with NT control. Mohr *et al.* (2013) have shown with the *ldh* knockout mutant a 4% reduction in lactate production in comparison with wild type. In contrast, we showed 24%

decrease in lactate production and 15% increase in formate; however, no increase in acetate or ethanol was detected. This was also noted with minor reduction in *pta* gene expression at exponential phase for both *ldh*_P and *ldh*_S. In another study, the *ldh* mutants of *H. thermocellum* were characterized, acetate titre was also not altered. However, no prominent difference was observed in gene expression and acetate production in the stationary phase. Thus, signifying lactate dehydrogenase deletion does not affect the flux through the pyruvate node if ethanol is produced (van der Veen *et al.*, 2013). Moreover, for ethanol production, there were no significant differences observed when compared to the NT control. This result was comparable to earlier reports of *H. thermocellum pta* mutant (Tripathi *et al.*, 2010). Future work in developing a *H. thermocellum* strain could involve *pta* and *ldh* multiplex silencing or knockout using dead or active thermophilic Cas9, respectively, to study if there is any impact on product formation. Nevertheless, the genetic system developed here demonstrates a step forward towards manipulating *H. thermocellum* strain with slight effects on other genes in the metabolic pathways.

The main challenge for applying thermophilic CRISPR-Cas system seems to be its robustness. In previous studies, this system has been successfully applied in *B. smithii* to reduce the highly expressed *ldhL* gene expression for about 90% resulting in 40% reduction in lactate production (Mougiakos *et al.*, 2017). Similarly, we have repressive effect for both *pta* and *ldh* genes with reduction in acetate and lactate, respectively. However, at stationary phase the cells overcome the repressive effect with less impact on product formation. This CRISPRi loss-of-function effect has been shown in several growth studies where essential genes were targeted (Zhao *et al.*, 2016; Liu *et al.*, 2017), correlating it with the presence of suppressor mutations in the dead Cas9 coding sequence at stationary phase. Even a single mutation of the first seven nucleotides intensely decreased repression, suggesting the importance of the 'seed region' sequence for binding of both the type I and type II CRISPR systems (Jinek *et al.*, 2012; Qi *et al.*, 2013). Therefore, it is likely that the importance of these metabolic genes pose a strong selection pressure for the cells that have overcome this burden by suppressor mutations for survival affecting the repression at stationary phase. Further studies are required to demonstrate that this is actually the case.

To date, *H. thermocellum* strains have been genetically engineered, improving the production of ethanol using cellulose as a carbohydrate source. Two main strategies have been followed. On one hand, competing pathways for carbon flux are disrupted (Argyros *et al.*, 2011; Biswas *et al.*, 2014; Papanek *et al.*, 2015; Kannuchamy *et al.*, 2016). In another approach, competing pathways for electron flux are eliminated (Biswas *et al.*, 2015; Rydzak *et al.*, 2015; Lo *et al.*, 2017). This is the first time a silencing tool has been applied to *H. thermocellum* DSM1313 to transcriptionally regulate the expression of genes involved in central metabolism. In this case, qRT-PCR has shown clear effect on the repression of both the genes in the exponential growth phase which leads to efficient redistribution of the organic acids and ethanol in *H. thermocellum* DSM1313.

Experimental procedures

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table S1, Supporting information. High efficiency NEB (New England Biolabs, CFU ~ 10^9 μg pUC19^{-1}) chemically competent *E. coli* DH5α was used for cloning purposes. *E. coli* Acella (Acella^{TM} Chemically Competent cells, Edge Bio) was used for plasmid propagation and isolation when *H. thermocellum* DSM 1313 was transformed to ensure the transfer of Dam-methylated plasmids. *E. coli* strains were cultured in Lysogeny-Broth (LB) medium (1% tryptone, 1% NaCl, 0.5% yeast extract) at 37°C and 200 rpm. When appropriate the medium was supplemented with 20 μg ml^{-1} chloramphenicol.

Hungateiclostridium thermocellum DSM 1313 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *H. thermocellum* DSM 1313 wild type and transformants were grown anaerobically in CFTÜD adapted from Olson and Lynd (2012) (0.13% ammonium sulphate, 0.15% potassium phosphate monobasic, 0.013% calcium chloride dehydrated, 0.05% L-cysteine hydrochloride, 1.15% MOPS sodium salt, 0.0001% ferrous sulfate heptahydrate, 0.26% magnesium chloride hexahydrate, 0.45% BD BBL™ Yeast Extract, 0.5% Na-resazurin solution 0.1% w/v and 0.3% sodium citrate tribasic dihydrate) broth and agar media at 50–60°C range (Olson and Lynd, 2012). For plasmid selection after electrotransformation, 6 μg ml^{-1} thiamphenicol was added. For RNA isolation and HPLC analysis, the silencing transformants were grown in CP medium adapted from Plugge, 2005 (0.408 g l^{-1} potassium dihydrogen phosphate, 0.534 g l^{-1} sodium phosphate dibasic dihydrate, 0.3 g l^{-1} ammonium chloride, 0.3 g l^{-1} sodium chloride, 0.1 g l^{-1} magnesium chloride hexahydrate, 0.11 g l^{-1} calcium chloride dihydrate, 4.0 g l^{-1} sodium bicarbonate, 1.0 g l^{-1} L-cysteine and 5.0 g l^{-1} (RNA isolation) or 1.0 g l^{-1} (HPLC) yeast extract (BD Bacto), 0.5 mg resazurin, 1 ml vitamin solution, 1 ml trace elements solution I and 1 ml trace elements solution II. The final volume of 50 ml medium was dispensed in serum bottles under 80:20 N_2/CO_2

atmosphere with ~70 kPa overpressure and then autoclaved. A stock solution comprising sodium bicarbonate and L-cysteine was autoclaved separately and added later. Likewise, vitamin stock solution was added to calcium chloride dihydrate solution after it was autoclaved. Cellobiose as a carbon source was also autoclaved separately and added later to a final concentration of 5.0 g l^{-1} (Plugge, 2005). The vitamin solution, 1000 × concentrated, contained per litre 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine-HCl, 50 mg thiamine-HCl, 50 mg riboflavin, 50 mg nicotinic acid, 50 mg Ca-D-pantothenate, 1 mg vitamin B12, 50 mg 4-aminobenzoid acid and 50 mg lipoic acid.Trace elements solution I, 1000 × concentrated, contained per litre 50 mM HCl, 61.8 mg H_3BO_4, 99.0 mg $MnCl_2{\cdot}4H_2O$, 1.49 g $FeCl_2{\cdot}4H_2O$, 119 mg $CoCl_2{\cdot}6H_2O$, 23.8 mg $NiCl_2{\cdot}6H_2O$, 68.2 mg $ZnCl_2$ and 17.0 mg $CuCl_2{\cdot}2H_2O$. Trace elements solution II, which was 1000 × concentrated, contained per litre 10 mM NaOH, 17.3 mg Na_2SeO_3, 33.0 mg $Na_2WO_4{\cdot}2H_2O$ and 24.2 mg $Na_2MoO_4{\cdot}2H_2O$.

CRISPRi plasmids construction

Both the plasmids and primers used in this study are listed in Table S2, Supporting information. Plasmids were built using fragments with the appropriated overhangs via NEBuilder® HiFi DNA assembly cloning kit according to the manufacturer's protocol. All PCR reactions for amplification of fragments were performed with the NEB Q5® High-Fidelity DNA polymerase (M0491). PCR fragments were subjected to 1% w/v agarose gel electrophoresis and isolated using Zymoclean™ Gel DNA Recovery kit. Plasmid pThermoCas9i (Mougiakos *et al*., 2017) was used as a template for the construction of all the plasmids. Plasmids were designed for the knockdown of the lactate dehydrogenase (*ldh*: Clo1313_1160) and phosphotransacetylase (*pta*; Clo1313_1185) in the genome of *H. thermocellum* DSM 1313. Two different protospacers were selected for each targeted gene and a non-targeting with random sequences was used as a control (Table S3, Supporting information). They differ on the position within the targeted gene. The target sites are the promoter, start of the gene and also the non-targeting spacer with random sequences. To block transcription, the non-template strand of the gene was targeted.Plasmids with alternative promoters to drive the sgRNA expression were based on the previously built ThermodCas9 plasmids by exchanging the *B. coagulans pta* promoter of the sgRNA expression module with 250 bp of the *H. thermocellum* intergenic region of the 16S and 23S rRNA genes (Table S1)**.** All plasmids were introduced by heat shock into chemically competent *E. coli* DH5α cells (Sambrook *et al*., 1989). Plasmids were isolated from selected single transformants by using the GeneJET Plasmid Miniprep Kit® (Thermo Scientific). Plasmid sequences were confirmed by standard sequencing from Macrogen (MACROGEN Inc. DNA Sequencing Service; Amsterdam, The Netherlands) using the primers present in Table S4, Supporting information. After sequence confirmation, plasmids were electroporated into *E. coli* Acella (Sambrook *et al*., 1989) before being introduced in *H. thermocellum* by electroporation as described by Olson and Lynd (2012).

RNA isolation

RNA isolation of *H. thermocellum* mutants, harbouring the pThermoCas9i plasmids, was performed using 5 ml of overnight cultures in the mid-log phase ($OD_{600\ nm}$ reached 0.6). In order to harvest the cells, the culture was brought inside the anaerobic chamber where 5 ml was transferred to a 50 ml Greiner tube. Cells were centrifuged aerobically at 4800 *g* and 4°C for 15 min. The supernatant was carefully decanted, and the pellet was resuspended in 0.5 ml of ice cold TE buffer (pH 8.0). The pellet was placed on ice during the next steps of the protocol, unless otherwise specified. The cell suspension was divided in two 2 screw-capped tubes containing 0.5 g of zirconium beads, 30 µl of 10% w/v SDS, 30 µl of 3 M sodium acetate (pH 5.2) and 500 µl of Roti® aqua Phenol (Carl Roth GmbH, Karlsruhe, Germany). Cells were disrupted in the Fastprep apparatus (MP Biomedicals, Solon, OH, USA) at speed 6 for 40 s, centrifuged at 10 000 rpm and 4°C, for 5 min. Three hundred microlitres of aqueous phase was transferred to a sterile 1.5 ml Eppendorf tube, and 300 µl of chloroform-isoamyl alcohol (Carl Roth GmbH, Karlsruhe, Germany) was added. The mixture was centrifuged at 14 000 rpm and 4°C, for 3 min. Two hundred and fifty microlitres of water phase was transferred to a sterile 1.5 ml Eppendorf tube and mixed with 250 µl of the Lysis/Binding buffer of the Maxwell® 16 LEV Total RNA Cells Kit. Consequently, the rest of the steps were followed according to the RNA isolation Kit. The purified RNA was measured in the NanoDrop spectrophotometer to determine the quality and the concentration of the elution and stored at −80°C.

First-strand cDNA synthesis and RT-PCR

The first-strand cDNA synthesis was performed in a 20-µl reaction, containing 2500 ng of RNA, 125 ng of random primers, 10 mM dNTP mix, 5× First-Strand Buffer, 0.1 M DTT, the SuperScript™ III RT and RNAse-free water, following the manufacturer's instruction of the SuperScript™ III Reverse Transcriptase protocol (Invitrogen (Life Technologies Europe BV), The Netherlands). The cDNA as

well as RNA was used in RT-PCR to analyse the expression of both, the ThermodCas9 protein and the sgRNA. The RNA was used as a negative control. The primers BG11642 and BG11643 were used to amplify 169 bp of the sgRNA using the NEB Q5® High-Fidelity DNA polymerase. The primers BG11636 and BG11637 were used to amplify 282 bp of the ThermodCas9 using the NEB Q5® High-Fidelity DNA polymerase.

Quantitative real time PCR

To assess the functionality of ThermodCas9 based CRISPRi as a silencing tool, a quantitative real-time PCR (RT-qPCR) was performed using the cDNA synthesized from the RNA of *H. thermocellum* DSM 1313, expressing the ThermodCas9 and the sgRNA targeting the lactate dehydrogenase (*ldh*) and phosphate acetyltransferase (*pta*) genes. As controls, the cDNA was also synthesized from the RNA of the wild-type *H. thermocellum* DSM 1313 strain and the transformant strains with ThermodCas9 and the non-targeting sgRNA. It was used to measure the relative expression level of the *ldh* and *pta* genes of the transformant *H. thermocellum*, comparing it to the transformant *H. thermocellum* with the ThermodCas9 and the non-targeting sgRNA.

RT-qPCR was performed by using the iQ™ SYBR® Green Supermix from Bio-Rad. The final volume of the reaction was set to 20 μl; thus, all the components were scaled accordingly relevant to the manufacturer's protocol. The cDNA samples were diluted in sterile Milli-Q water. The amount of cDNA used as a template was relative to 50 ng of RNA. The house-keeping gene used to measure the relative expression was the recombinase A (*recA*) of *H. thermocellum* DSM 1313. The primers used to amplify the *ldh* and *pta* genes of *H. thermocellum* DSM 1313 were BG14575, BG14580 and BG15853, BG15854 respectively (Table S6, Supporting information). The RT-qPCR was run in a Bio-Rad C1000 Thermal Cycler.

High-pressure liquid chromatography

A high-pressure liquid chromatography (HPLC) system ICS-5000 was used for the organic acids and ethanol quantification. The system has Aminex HPX 87H column from Bio-Rad Laboratories and is equipped with a UV1000 detector operating on 210 nm and a RI-150 40 °C refractive index detector. The mobile phase consisted of 0.16 N H_2SO_4, and the column was operated at 0.8 ml min^{-1}. All samples were diluted 4:1 with 10 mM DMSO in 0.01 N H_2SO_4. The *H. thermocellum* NT control, *ldh* and *pta* silencing transformant strains were grown in CP medium (1 g l^{-1} yeast extract) for 2 days and samples were taken at different time points from $OD_{600} \sim 0.5–1.5$ to analyse using HPLC. Sugars (cellobiose, glucose, ethanol and glycerol) and organic acids (acetic acid, lactic acid, succinic acid and formic acid) were used as standards with a concentration range between 1.25 and 25 mM.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. For lactate and acetate production, positive transformants referred as *ldh*_P, *ldh* _S, *pta*_P, *pta*_S and control *ldh*_non-targeting (NT) were analyzed in HPLC at $OD_{600} > 1.0$ (T2). The silencing transformants for *ldh* gene referred as *ldh*_P – targeting the promoter, *ldh* _S – targeting the start of the gene; positive silencing transformants for *pta* gene referred as *pta*_P – targeting the promoter, *pta*_S – targeting the start of the gene. *H. thermocellum* DSM 1313 cells were transformed with both non-targeting pThermoCas9i_NT and targeting pThermoCas9i_*ldh* and *pta* plasmids. The Tm^R colonies of both the non-targeting and targeting plasmids were transferred to 50 ml CP medium (1g l^{-1} yeast extract) containing 6 μg ml^{-1} Tm^R and cultured to $OD_{600} \sim 0.5–0.7$ (T1) and > 1.0 (T2), and 1 ml cells were sampled for 2 days for HPLC analysis. Data represent the mean values of three biological replicates and the standard deviation (SD). The level of significance of the differences when results were compared was estimated by means of analysis of variance (ANOVA), with $\alpha = 0.05$.

Table S1. Bacterial strains used in the present study.

Table S2. Plasmids used in the present study.

Table S3. Spacers used in the present study.

Table S4. Primers used in the present study.

Appendix S1. Promoter intergenic 16S/23S rRNA_C. thermocellum DSM1313.

Appendix S2. Effects of CRISPRi-mediated suppression on lactate and acetate production and impact on the fermentation profile.

